

# **EXHIBIT 17**

# Viral Infection Causes Rapid Sensitization to Lipopolysaccharide: Central Role of IFN- $\alpha\beta$ <sup>1</sup>

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LPS is the major active agent in the pathogenesis of Gram-negative septic shock. In this report we have studied the influence of concurrent viral infection on the outcome of LPS-induced shock. We find that infection with vesicular stomatitis virus sensitizes mice to LPS at an early time point following infection. Treatment of mice with the chemical IFN inducer, polyinosinic:polycytidylic acid, has a similar effect. This hypersensitivity to LPS correlated with hyperproduction of TNF- $\alpha$  in vivo. The cellular and molecular mechanisms underlying this phenomenon were investigated using Ab-depleted and gene-targeted mice. Our results revealed that while NK cell depletion and elimination of IFN- $\gamma$  partially protected against the sensitizing effects of vesicular stomatitis virus and polyinosinic:polycytidylic acid, the most striking effect was observed in IFN- $\alpha\beta$ -deficient mice. Thus hyperproduction of TNF- $\alpha$  was completely abrogated in IFN- $\alpha\beta$ -deficient mice, indicating that the principal mechanism underlying rapid virus-induced sensitization to LPS is an IFN- $\alpha\beta$ -mediated priming of mice for an augmented production of TNF- $\alpha$  in response to LPS. This conclusion was further supported by the finding that pretreatment of mice with rIFN- $\alpha\beta$  mimicked the effect of viral infection. In conclusion, our results reveal a previously unrecognized proinflammatory effect of IFN- $\alpha\beta$  and point to a new pathway through which viral infection may influence the outcome of concurrent bacterial infection. *The Journal of Immunology*, 2001, 166: 982–988.

**T**he Gram-negative bacterial wall constituent, endotoxin (LPS), is the major active agent in the pathogenesis of septic shock. A shock-like state can be induced by a single injection of LPS into animals (1). Most of the toxic effects of LPS are mediated through the release and action of macrophage-derived inflammatory cytokines (1–3). TNF- $\alpha$  appears to constitute a central element in the pathogenesis as indicated by the relative resistance to LPS-induced toxicity in mice lacking the p55 TNFR, the TNF- $\alpha$  molecule, or producing high levels of soluble TNFR1 fusion protein (4–6).

Another important regulator of LPS-induced pathology is IFN- $\gamma$  (3, 7), the involvement of which is supported by several lines of evidence. Administration of IFN- $\gamma$  or neutralizing Abs to IFN- $\gamma$  has been shown to modify the lethal outcome of several forms of endotoxic shock and Gram-negative bacterial infections, and experiments with IFN- $\gamma$ -deficient mice have revealed that these mice are relatively resistant to LPS-induced shock (2, 8, 9). It is generally believed that one aspect of the contribution of IFN- $\gamma$  in LPS-induced shock consists in priming monocytes/macrophages by inducing the expression of receptors for TNF- $\alpha$  on the surface of these cells, which in turn enables autocrine binding of TNF- $\alpha$  and subsequent activation of monocytes/macrophages (10–13). In addition to the early effect exerted by IFN- $\gamma$  in LPS-induced

shock, IFN- $\gamma$  also seems to promote LPS-induced lethality by more late-acting mechanisms (2). In mice, a generalized shock syndrome, known as the generalized Shwartzman reaction, can be elicited by two consecutive injections of LPS (14, 15). A priming dose of LPS is injected into the footpad and followed after 24 h by an i.v. challenge injection of LPS. After the last challenge, which is nonlethal per se, the mice develop a generalized shock syndrome and die within 48 h. IFN- $\gamma$  has been shown to be a critical component in the priming phase of the generalized Shwartzman reaction. Thus local injection of LPS is believed to trigger release of IL-12 and IL-15 that synergizes in inducing NK cells to produce IFN- $\gamma$  (16), which in turn primes macrophages for activation. Upon subsequent exposure to LPS, the primed macrophages become hyperactivated and produce large amounts of TNF- $\alpha$  and IL-1 (17, 18). Thus endogenous IFN- $\gamma$  may sensitize mice to otherwise nonlethal doses of LPS.

The involvement of IFN- $\gamma$  in the pathogenesis of endotoxic shock prompted us in a previous study to investigate whether production of this cytokine in the context of viral infection could alter antibacterial host responses through a modified cytokine network (19). Our results revealed that systemic infection of mice with the noncytopathogenic lymphocytic choriomeningitis virus (LCMV)<sup>3</sup> sensitized mice to low amounts of LPS, and that this hypersensitivity correlated with hyperproduction of TNF- $\alpha$ . Hyperproduction of TNF- $\alpha$  was found to be temporally correlated with virus-induced T cell-dependent production of IFN- $\gamma$ , thus only a marginally increased IFN- $\gamma$  and TNF- $\alpha$  production was observed in T cell-deficient nude (*nu/nu*) mice and in mice infected with vesicular stomatitis virus (VSV), a virus that induces less extensive T cell activation than does LCMV. Furthermore, LCMV infection was found to be much less efficient in priming IFN- $\gamma$ -deficient mice for hyperproduction of TNF- $\alpha$  (19), and neutralization of the latter cytokine markedly protected against a lethal outcome (20). Notably, however, our

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<sup>3</sup> Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; poly(I:C), polyinosinic:polycytidylic acid; VSV, vesicular stomatitis virus; *nu/nu*, nude;  $\mu$ MT/ $\mu$ MT, B cell deficient; rHuIFN- $\alpha$ , recombinant human hybrid IFN- $\alpha$  A/D.

results revealed that LPS-induced production of TNF- $\alpha$  was significantly augmented in both LCMV-infected T cell-deficient *nu/nu* mice and IFN- $\gamma$ -deficient mice compared with levels found in matched uninfected controls. Moreover, very similar results were obtained when wild-type mice were challenged with LPS at an early time-point after LCMV infection, before T cells have been substantially activated. Together, these findings suggest that although T cell-dependent IFN- $\gamma$  production is important, it is not the only mechanism through which viruses may prime for TNF- $\alpha$  production. IFN- $\alpha\beta$  is a likely candidate in this respect, because most viral infections including LCMV and VSV are known to induce substantial production of IFN- $\alpha\beta$  (21–25). In virus-infected immunocompetent mice, production of IFN- $\alpha\beta$  generally reaches peak levels 1–3 days post infection (p.i.), and IFN- $\alpha\beta$  has been shown to be a critical and indispensable component of early antiviral host defenses as mice made genetically deficient for IFN- $\alpha\beta$ R expression are often unable to control virus replication, and may die (24).

IFN- $\alpha\beta$  has been found to synergize in vitro with subactivating doses of LPS to activate macrophages for production of TNF- $\alpha$  and to enhance their microbicidal activity (26, 27). Therefore, the present study was undertaken to explore in greater detail whether early virus-induced production of IFN- $\alpha\beta$  could sensitize mice to LPS-induced shock through an augmented production of TNF- $\alpha$  in response to LPS. This was done by monitoring production of TNF- $\alpha$  and lethality following an i.p. injection of LPS into mice preinfected with VSV or pretreated with the chemical IFN-inducer polyinosinic:polycytidylic acid (poly(I:C)) (28, 29). The response of a number of mutant mice with targeted defects in genes of potential interest was analyzed and compared with wild-type mice. We demonstrate in this study that virus-induced production of IFN- $\alpha\beta$  may prime mice for an augmented production of TNF- $\alpha$  in response to LPS. This is evidenced by the findings that an augmented production of TNF- $\alpha$  occurs despite the lack of either NK cells, T cells, B cells, or IFN- $\gamma$ , but is completely abolished in mice made genetically deficient for IFN- $\alpha\beta$ R expression. Furthermore, pretreatment of mice with a rIFN- $\alpha\beta$  also augmented TNF- $\alpha$  production and increased the lethality. Thus this seems to be a general phenomenon that may be induced by different viral infections and, therefore, may be regarded as a virus-induced analogue to the bacterially induced generalized Shwartzman reaction.

## Materials and Methods

### Mice

C57BL/6, C57BL/6-*nu/nu*, and 129/Sv mice were obtained from Bomholtgaard (Ry, Denmark). Mice defective in IFN- $\alpha\beta$ R expression (IFN- $\alpha\beta$ R<sup>-/-</sup>) on a 129 background were derived from breeding pairs from B & K Universal (North Humberside, U.K.). Mice deficient in production of IFN- $\gamma$  (IFN- $\gamma$ <sup>-/-</sup>) and B cell deficient ( $\mu$ MT/ $\mu$ MT) mice both on a C57BL background were the progeny of breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME) and the National Institutes of Health (Bethesda, MD), respectively.  $\mu$ MT/ $\mu$ MT mice were bred using heterozygous female and homozygous males, and the offspring were selected by testing sera in a sandwich ELISA for the presence of IgM Abs. Unless otherwise specified, 6- to 8-wk-old female mice were used in all experiments, and animals from outside sources were always allowed to acclimatize to the local environment for at least 1 wk before use. All animals were housed under specific pathogen-free conditions as validated by testing of sentinels for unwanted infections according to Federation of European Laboratory Animal Science Association guidelines; no such infections were detected.

### Infection/pretreatment of mice

Where indicated, mutant and wild-type mice were infected with VSV of the Indiana strain originally provided by K. Berg of the Institute of Medical Microbiology and Immunology (Copenhagen, Denmark), and in one experiment with LCMV of the Traub strain (30). In other experiments, mutant and wild-type mice were treated with poly(I:C) (Sigma, St. Louis,

MO). Mice to be infected received a virus dose of  $10^3$  LD<sub>50</sub> LCMV or  $10^6$  PFU VSV in an i.v. injection of 300  $\mu$ l, whereas mice to be treated with poly(I:C) were injected i.p. with 150  $\mu$ g poly(I:C) dissolved in 150  $\mu$ l sterile PBS.

### Recombinant human hybrid IFN- $\alpha$ A/D (rHuIFN- $\alpha$ )

rHuIFN- $\alpha$  originating from Hoffmann-La Roche (Nutley, NJ) was kindly provided by K. Berg (Institute of Medical Microbiology and Immunology). This rHuIFN- $\alpha$  has previously been tested in mice and found to exert the expected effect (31, 32). Mice to be treated received  $2 \times 10^6$  U i.p. the day before LPS challenge.

### In vivo depletion of NK cells

Mice were depleted of NK cells 1 day before treatment with poly(I:C) or infection with VSV. NK cells were depleted by an i.v. injection of 50  $\mu$ l purified rabbit anti-asialo G<sub>M1</sub> Abs (Wako Pure Chemicals, Osaka, Japan) diluted in 300  $\mu$ l PBS. Control mice received an i.v. injection of 300  $\mu$ l PBS. NK cell depletion was confirmed by testing NK cell cytotoxic activity in a standard (51) Cr release assay, using NK cell sensitive YAC-1 cells as targets (30).

### LPS challenge

Mice were challenged i.p. with LPS from *Escherichia coli* serotype 055:B5 (Sigma), and unless otherwise specified, the challenge dose was 50  $\mu$ g/mouse. Virus-infected mice were challenged with LPS on day 1 p.i. (VSV) and in one experiment on day 8 p.i. (LCMV), whereas mice injected with poly(I:C) were challenged with LPS 1 day after treatment. Control groups of mice were included in all experiments and consisted of mice that received LPS alone or were injected i.p. with 150  $\mu$ l PBS 1 day before LPS challenge (poly(I:C) experiments).

### Lethality experiments

In most experiments mortality was recorded 24 and 48 h after LPS challenge.

### Quantitation of cytokine levels in serum

Cytokine concentrations in sera were determined using a sandwich ELISA. The following ELISA kits (Endogen, Cambridge, MA) were used in this study: TNF- $\alpha$ , IL-6, IL-1 $\alpha$ , and IL-1 $\beta$ . The assays were run according to the manufacturer's instructions, and cytokine levels in sera were calculated by comparison with a standard curve generated using recombinant cytokine; the limit of detection for all cytokines was 15 pg/ml.

## Results

### Sensitization to LPS in VSV-infected and poly(I:C)-treated animals

It has previously been found that LCMV infection is associated with markedly increased sensitivity to LPS (19, 20). Although the infected mice were most susceptible to LPS during the height of the adaptive response, significantly increased lethality was observed already during the innate response. To examine whether rapid sensitization to LPS could be induced by viruses other than LCMV, we examined the susceptibility to LPS of mice infected with VSV or treated with poly(I:C). Briefly, lethality experiments were conducted in mice infected with VSV or treated with poly(I:C) 1 day before LPS challenge. All mice that were challenged with LPS received an i.p. challenge dose of 50  $\mu$ g/mouse, and mortality was recorded after 24 and 48 h (Table I).

All mice infected with VSV 1 day before LPS challenge died within 24 h and very similar results were obtained in mice injected with poly(I:C) 1 day before LPS challenge; approx. 2/3 of these mice died within 24 h, and the remaining died within 48 h. None of the mice that were infected with VSV, treated with poly(I:C), or challenged with LPS alone died during the 48-h observation period.

The outcome of these lethality experiments together with the previous finding that mice infected with LCMV 3 days before LPS

Table I. Sensitization to LPS in mice treated with poly(I:C) or infected with VSV

Treatment <sup>a</sup>		TNF- $\alpha$ (ng/ml) <sup>b</sup>	Mortality, No. Dead/Total (%) <sup>c</sup>	
			24 h	48 h
Poly(I:C)	LPS	151.6	15/22 (68)	22/22 (100)
PBS	LPS	25.9	0/14 (0)	0/14 (0)
Poly(I:C)	None	<1.8	0/3 (0)	0/3 (0)
VSV	LPS	143.1	6/6 (100)	6/6 (100)
None	LPS	9.1	0/6 (0)	1/6 (17)
VSV	None	<0.3	0/6 (0)	0/6 (0)

<sup>a</sup> Mice were treated i.p. with 150  $\mu$ g poly(I:C) or infected i.v. with VSV ( $2 \times 10^6$  PFU) 1 day before challenge with 50  $\mu$ g LPS/mouse.

<sup>b</sup> Animals were bled 1.5 h after challenge with LPS for determination of TNF- $\alpha$  serum levels. TNF- $\alpha$  levels shown are medians of the respective groups.

<sup>c</sup> Mortality was recorded 24 and 48 h after LPS challenge.

challenge also succumb within 48 h, strongly suggest that early virus-induced sensitization to LPS is a general phenomenon that may be induced by different viral infections.

#### Production of TNF- $\alpha$ in VSV-infected and poly(I:C)-treated mice after LPS challenge in vivo

To see whether the enhanced sensitivity to LPS in VSV-infected and poly(I:C)-treated mice was reflected in the production of TNF- $\alpha$ , serum levels of TNF- $\alpha$  were determined 1.5 h after LPS challenge (Table I). As expected, augmented serum levels of TNF- $\alpha$ , which clearly exceeded those found in mice infected with VSV, treated with poly(I:C), or challenged with LPS alone, were detected in mice infected with VSV or treated with poly(I:C) 1 day before LPS challenge. Thus enhanced susceptibility to LPS-induced shock correlated with a 5- to 10-fold increase in the production of TNF- $\alpha$  in virus-infected or poly(I:C)-treated mice. In contrast, we did not find the production of IL-1 $\alpha$ , IL-1 $\beta$ , or IL-6 to be substantially augmented (<2-fold).

#### Comparison between early and late virus-induced sensitization to LPS

To characterize this rapidly induced sensitization to LPS in greater detail, we compared the response to LPS at this stage to the late LCMV-induced sensitization to LPS in which situation the most important priming mechanism has recently been shown to be the production of IFN- $\gamma$  by virus-activated T cells (19, 20). Briefly, LPS-induced mortality and production of TNF- $\alpha$  in response to two different doses of LPS were compared between mice treated with poly(I:C) 1 day before LPS challenge and mice infected with LCMV 8 days before LPS challenge reflecting the time-point of maximal T cell activation (Fig. 1). A clearly dose-dependent production of TNF- $\alpha$  was observed in both poly(I:C)-treated mice and LCMV-infected mice. Furthermore, irrespective of the LPS challenge dose used, serum levels of TNF- $\alpha$  were clearly elevated in mice treated with poly(I:C) or infected with LCMV compared with the levels measured in mice that received LPS alone. However, the serum levels of TNF- $\alpha$  in response to either challenge dose of LPS were generally three to five times lower in mice treated with poly(I:C) than in mice infected with LCMV 8 days earlier. With respect to LPS-induced mortality, our experiments revealed that mice treated with poly(I:C) 1 day before LPS challenge died within 48 h when challenged with 50  $\mu$ g LPS, but survived challenge with 2  $\mu$ g LPS. In contrast, all mice infected 8 days earlier with LCMV died within 24 h when challenged with either dose of LPS; none of the mice challenged with LPS alone died.

From the results outlined above it is evident that mice are less susceptible to LPS during the innate phase of the host response than seen later in the infection; thus a relatively high challenge dose of LPS was required to elicit lethal amounts of TNF- $\alpha$  in

mice treated with poly(I:C). Nevertheless, poly(I:C) did prime mice for an augmented production of TNF- $\alpha$  even in response to the lower LPS dose.

#### The role of NK cells

Production of IFN- $\gamma$  by NK cells has been shown to play an essential role in lethal LPS-induced Shwartzman reaction in mice (18). Moreover, during the course of a viral infection IFN- $\gamma$  may be produced not only by virus-activated T cells but also by virus-activated NK cells (33, 34), and NK cell activation can be observed in mice infected with VSV or pretreated with poly(I:C) as evidenced by augmented ex vivo cytotoxicity (data not shown). To find out whether early virus-induced priming of mice for an augmented production of TNF- $\alpha$  was dependent on NK cells, we examined the susceptibility to LPS in mice depleted of NK cells and either preinfected with VSV or pretreated with poly(I:C) 1 day before LPS challenge. As shown in Fig. 2, depletion of NK cells did not eliminate VSV- and poly(I:C)-dependent sensitization for an augmented production of TNF- $\alpha$ . Partial protection against a lethal outcome was observed; this may reflect both the reduced TNF- $\alpha$  response in some mice as well as a role for NK cells in later stages of LPS-induced shock.

#### The role of T cells

It has previously been found that both viruses and poly(I:C) can activate CD44<sup>high</sup> T cells, a subset characterized by the capacity to

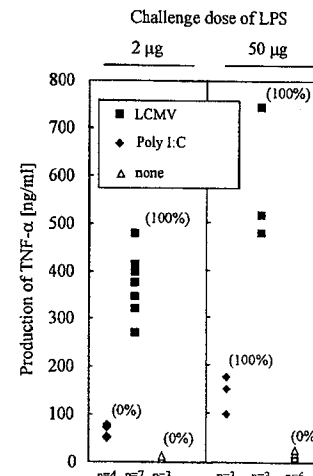
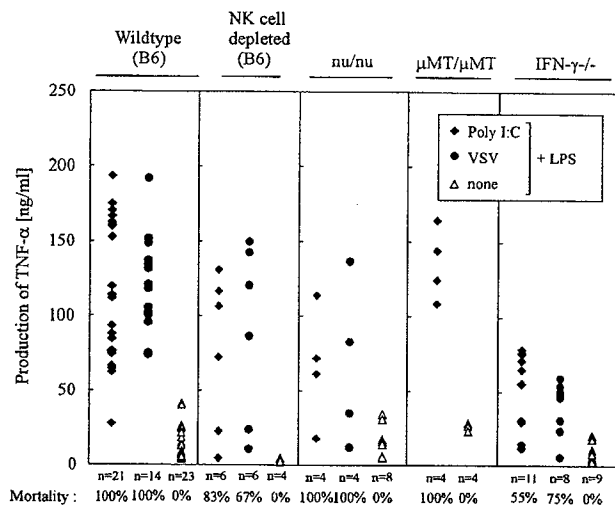


FIGURE 1. Comparison between early and late virus-induced sensitization to LPS. Groups of B6 mice were injected i.p. with poly(I:C) 1 day before LPS challenge, infected i.v. with LCMV 8 days before LPS challenge, or left untreated. Mice were challenged with either 2 or 50  $\mu$ g LPS/mouse and the serum levels of TNF- $\alpha$  were determined 1.5 h after challenge with LPS. The shapes represent individual mice.



**FIGURE 2.** The role of NK cells, T cells, B cells, and IFN- $\gamma$  in early virus-induced sensitization to LPS. Wild-type (B6), NK cell-depleted (B6), T cell-deficient (*nu/nu*), B cell-deficient ( $\mu$ MT/ $\mu$ MT), and IFN- $\gamma$ -deficient (IFN- $\gamma$ <sup>-/-</sup>) mice were injected i.p. with poly(I:C) 1 day before LPS challenge or infected i.v. with VSV 1 day before LPS challenge, respectively. Mice were challenged with 50  $\mu$ g LPS/mouse. Control groups consisted of wild-type or mutant mice that received LPS alone. Serum levels of TNF- $\alpha$  were determined 1.5 h after challenge with LPS; the shapes represent individual mice. Mortality was registered for 48 h after LPS challenge.

produce IFN- $\gamma$  (35, 36). To examine the possible influence of early T cell-dependent production of IFN- $\gamma$  in the early sensitization to LPS, we evaluated the susceptibility to LPS in T cell-deficient *nu/nu* mice infected with VSV or treated with poly(I:C) 1 day before LPS challenge and in *nu/nu* mice challenged with LPS alone, and compared the observed responses to those in corresponding groups of wild-type mice (Fig. 2). Although the priming effect was less consistent in *nu/nu* mice than in similarly treated wild-type animals, the overall picture is clearly that T cells are not mandatory. We assume that the greater variability in the response of *nu/nu* mice reflects the well-known tendency toward an altered stage of macrophage activation in these mice.

#### The role of B cells

LPS (endotoxin) is well known for its capacity to activate B cells that may produce IFN- $\gamma$  under certain conditions (37). To examine whether early virus-induced priming of mice for an increased susceptibility to LPS involved B cells, we examined the response of B cell-deficient  $\mu$ MT mice treated with poly(I:C) 1 day before LPS challenge with respect to lethality and production of TNF- $\alpha$ . As shown in Fig. 2, the LPS-induced production of TNF- $\alpha$  was as high in poly(I:C)-treated  $\mu$ MT mice as in wild-type mice, and all pretreated mice succumbed to the LPS challenge. Thus early virus-induced sensitization to LPS does not require the presence of B cells.

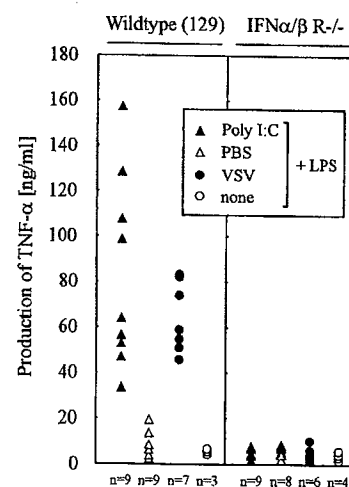
#### The role of IFN- $\gamma$

IFN- $\gamma$  is considered to be the most potent macrophage-activating cytokine and may be produced by many cell types. To determine the role of IFN- $\gamma$  in early virus-induced sensitization to LPS, we examined the susceptibility to LPS in IFN- $\gamma$ -deficient (IFN- $\gamma$ <sup>-/-</sup>) mice treated with poly(I:C) or infected with VSV 1 day before LPS challenge. As shown in Fig. 2, IFN- $\gamma$ <sup>-/-</sup> mice were primed for an augmented production of TNF- $\alpha$  and most succumbed to LPS challenge when pretreated with poly(I:C) or infected with VSV. However, TNF- $\alpha$  levels in serum were generally lower than found

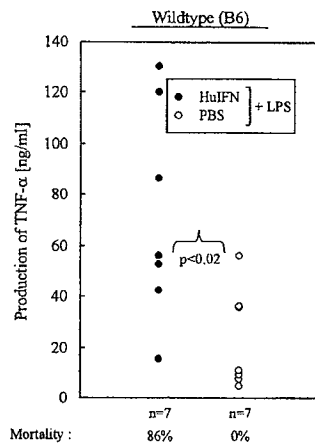
in similarly pretreated wild-type mice. This finding is consistent with the results obtained following NK cell depletion and indicate that NK cell produced IFN- $\gamma$  augments the early sensitization to viral infection although this cytokine is not absolutely essential. In this respect it may be important to note that the relative resistance of IFN- $\gamma$ R-deficient mice to LPS-induced shock has been shown to reflect a diminished expression of receptors for LPS on monocytes and macrophages in these mutants (9). As both poly(I:C) and VSV are allowed only a short action time in our experimental setup, we cannot rule out the possibility that the defect in IFN- $\gamma$ <sup>-/-</sup> mice partially reflects deficient expression of LPS receptors on the surface of monocytes and macrophages causing a generally reduced responsiveness toward LPS.

#### The role of IFN- $\alpha\beta$

A common denominator of viral infection and treatment with poly(I:C) is rapid induction of high amounts of IFN- $\alpha\beta$ . In vitro, endogenous IFN- $\alpha\beta$  has previously been shown to synergize in an autocrine manner with subactivating doses of LPS to activate macrophages for production of TNF- $\alpha$  and to enhance their microbicidal activity (27). Furthermore, in a recent in vivo study it was shown that regulation of type 2 NO synthase expression during the innate immune response in mice to *Leishmania major* is dependent on IFN- $\alpha\beta$  (38). Therefore, to investigate whether IFN- $\alpha\beta$  was pivotal for the early virus-induced increase in susceptibility to LPS, we examined the priming effect of poly(I:C) and VSV in IFN- $\alpha\beta$ R-deficient mice (IFN- $\alpha\beta$ R<sup>-/-</sup>) (Fig. 3). Because this genetic defect has not been bred onto a C57BL/6 background, we had to use mice of the 129 Sv strain for this part of our analysis. As wild-type (129) mice were found to be substantially more sensitive to the toxicity of LPS (also unprimed mice died within 48 h), lethality was not well suited as a discriminating parameter although we did consistently see accelerated mortality following priming. However, analysis of TNF- $\alpha$  levels gave an unequivocal readout. Thus as previously seen with C57BL/6 mice, wild-type (129) mice were primed for an augmented TNF- $\alpha$  production in response to LPS when treated with poly(I:C) or infected with VSV 1 day before LPS challenge. In contrast, the priming capacity of



**FIGURE 3.** The role of IFN- $\alpha\beta$  in early virus-induced sensitization to LPS. Wild-type (129) mice and IFN- $\alpha\beta$ R-deficient mice (IFN- $\alpha\beta$ R<sup>-/-</sup>) were injected i.p. with poly(I:C) or infected with VSV 1 day before LPS challenge. Control groups of wild-type (129) mice or IFN- $\alpha\beta$ R<sup>-/-</sup> mice were injected i.p. with PBS 1 day before LPS challenge (poly(I:C)) or left untreated (VSV). Mice were challenged with 50  $\mu$ g LPS/mouse. Serum levels of TNF- $\alpha$  were determined 1.5 h after challenge with LPS; the shapes represent individual mice.



**FIGURE 4.** Pretreatment with rHuIFN- $\alpha$  sensitizes mice to LPS. Wild-type (C57BL/6) mice were given either  $2 \times 10^6$  units of rHuIFN- $\alpha$  or PBS i.p. 1 day before LPS challenge ( $50 \mu\text{g}/\text{mouse}$ ). Serum levels of TNF- $\alpha$  were determined 1.5 h after challenge with LPS; the shapes represent individual mice. Mortality was registered for 48 h after LPS challenge. The difference between IFN- $\alpha$ -pretreated and sham-treated mice was statistically significant with regard to both parameters ( $p < 0.02$ ; Mann-Whitney rank sum test for cytokine levels and Fisher's exact test for mortality).

both poly(I:C) and VSV was completely abolished in IFN- $\alpha\beta$  $^{-/-}$  mice and the production of TNF- $\alpha$  in pretreated/infected IFN- $\alpha\beta$  $^{-/-}$  mice did not exceed the production induced in mutant mice challenged with LPS alone. This result clearly demonstrates that early virus-induced priming for an augmented production of TNF- $\alpha$  in response to LPS requires IFN- $\alpha\beta$ . To our knowledge this in vivo priming capacity of IFN- $\alpha\beta$  for production of TNF- $\alpha$  has not been described before.

#### *Pretreatment with rIFN- $\alpha$ sensitizes mice to LPS induced shock*

Finally, to test whether IFN- $\alpha\beta$  sufficed for an augmented response to LPS, wild-type (C57BL/6) mice were pretreated with rHuIFN- $\alpha$  to which murine cells are responsive (31, 32, 39); a matched control group received PBS only. Twenty-four hours later all mice were challenged with LPS and 1.5 h later TNF- $\alpha$  levels in serum were analyzed, and mortality over the next 48 h was registered. As evident from Fig. 4, pretreatment with rIFN- $\alpha$  significantly increased both LPS-induced TNF- $\alpha$  production and mortality.

## Discussion

In this report we have demonstrated and analyzed virus-induced sensitization to LPS resulting from triggering of innate defense mechanisms. We find that viral infection as well as a well known IFN inducer substantially increases the sensitivity of mice to the toxic effects of LPS. A central mediator in this sensitization is IFN- $\alpha\beta$  as evidenced by the following observations. First, as already mentioned pretreatment of mice with the IFN-inducer poly(I:C) mimics the effect of viral infection. Second, priming is completely abolished in IFN- $\alpha\beta$  $^{-/-}$  mice. Finally, pretreatment with rHuIFN- $\alpha$  known to work on murine cells also increases the sensitivity to LPS. Notably, IFN- $\gamma$  was not essential for early virus-induced sensitization to LPS, demonstrating that the mechanism underlying this phenomenon differs from the mechanism underlying late virus-induced sensitization to LPS, which we have shown to be dependent on production of IFN- $\gamma$  by virus-activated T cells (19). A common feature of either scenario is the priming of virus-infected mice for an augmented production of TNF- $\alpha$  in response to LPS and an increased susceptibility to endotoxic shock.

The increased susceptibility to LPS in virus-infected and poly(I:C)-treated mice as revealed in lethality experiments always correlated with an augmented production of TNF- $\alpha$ . Given the central role of TNF- $\alpha$  in LPS-induced shock, and the fact that injection of this cytokine causes an essentially identical syndrome (40, 41), it is reasonable to assume that the augmented production of TNF- $\alpha$  plays an important pathogenic role. However, this does not exclude the critical participation of other cytokines in virus-induced sensitization to LPS either during the priming phase or as comediators.

Production of IFN- $\gamma$  by NK cells has been shown to play an essential role in the lethal LPS-induced Shwartzman reaction in mice (18), and recently production of IFN- $\gamma$  by NK cells has also been shown to be implicated in virus-induced sensitization to LPS (20). In the present set-up, depletion of NK cells did not eliminate the priming effect on LPS-induced production of TNF- $\alpha$ , indicating that production of IFN- $\gamma$  by NK cells is not essential for early virus-induced priming of mice for an augmented production of TNF- $\alpha$ .

IFN- $\alpha\beta$  has also been shown to activate CD44<sup>high</sup> T cells that are known to have a high capacity for production of IFN- $\gamma$  (35, 36). Our experiments with *nu/nu* mice revealed that early virus-induced priming for an enhanced production of TNF- $\alpha$  may occur despite the lack of functional T cells. The results obtained with NK cell-depleted and *nu/nu* mice were both supported by the finding that IFN- $\gamma$  $^{-/-}$  mice produced enhanced amounts of TNF- $\alpha$  when infected with VSV or treated with poly(I:C). However, we did find some reduction in the amounts of TNF- $\alpha$  produced in primed IFN- $\gamma$  $^{-/-}$  mice compared with similarly treated wild types. This could suggest that IFN- $\gamma$  augmented IFN- $\alpha\beta$ -dependent priming. Alternatively, deficient expression of relevant receptors for LPS may limit the general sensitivity in these mice thus reducing the basic set point for TNF- $\alpha$  production (9).

B cells may proliferate in vivo in response to LPS and may produce IFN- $\gamma$  in vivo when stimulated with IL-18 and IL-12 (37). Our experiments revealed that B cells are not required in early virus-induced sensitization to LPS because poly(I:C)-treated  $\mu\text{MT}/\mu\text{MT}$  mice were primed for an augmented LPS-induced production of TNF- $\alpha$ .

In the generalized Shwartzman reaction it is well established that the local injection of LPS leads to production of IFN- $\gamma$  by NK cells. IFN- $\gamma$  then primes macrophages for activation, and upon subsequent exposure to LPS the primed macrophages become hyperactivated and produce enhanced amounts of TNF- $\alpha$  (18). In early virus-induced sensitization to LPS, IFN- $\alpha\beta$  could partly work by inducing NK cells to produce IFN- $\gamma$ . However, because absence of NK cells and IFN- $\gamma$  did not prevent the priming effect of viral infection, other mechanism must also be important. Therefore, it is likely that macrophages are also primed by IFN- $\alpha\beta$  in an autocrine manner. This assumption is supported by in vitro studies. Thus, Influenza A infected human macrophages have been shown to produce dramatically elevated levels of TNF- $\alpha$  when cocultured with LPS, and because infected macrophages produced IFN- $\alpha\beta$ , the authors concluded that priming of infected macrophages by this cytokine preconditioned these cells to respond to LPS (42). Moreover, macrophage-synthesized IFN- $\alpha\beta$  can augment NO production in an autocrine fashion in cultures stimulated with subactivating doses of LPS (26, 42). In line with these observations a recent in vivo study has revealed that IFN- $\alpha\beta$  regulates early NO production in *Leishmania major*-infected mice and that this effect is important for parasite containment (38).

The mechanism of IFN- $\alpha\beta$ -mediated priming in vivo is likely to involve the Kupffer cells of the liver, which comprises the largest fixed macrophage population in the mammalian body. Kupffer

cells have been shown in vitro to produce TNF- $\alpha$  in response to endotoxin and are also able to produce IFN- $\alpha\beta$  (43). However, by use of the RNase protection assay we have not been able to convincingly demonstrate increased levels of mRNA coding for any of the major proinflammatory cytokines including TNF- $\alpha$  in the liver and spleen of mice treated with poly(I:C) 1 day earlier (data not shown). Therefore, rather than acting as a promoter of transcription of proinflammatory cytokines, IFN- $\alpha\beta$ -mediated priming may act as to increase LPS responsiveness of macrophages by inducing receptors for TNF- $\alpha$  and LPS (CD14) on the surface of these cells. This assumption is supported by the finding that elevated serum levels of soluble TNF- $\alpha$ R type II can be detected in wild-type mice, but not in IFN- $\alpha\beta$ R<sup>-/-</sup> mice, during the innate host response toward different viral infections (44).

The proinflammatory action of IFN- $\alpha\beta$  described in this report seemingly contradicts previous studies in which IFN- $\alpha\beta$  has been reported to down-regulate inflammatory responses to LPS. Systemic administration of rIFN- $\alpha$  20 min after i.p. challenge of mice with a lethal dose of LPS reduced the LPS-induced mortality by almost 90% and interestingly, some protection was observed even if rIFN- $\alpha$  was administered 1 h before LPS challenge (43). IFN- $\alpha\beta$  has also been reported to down-regulate local inflammation, thus the development of the local LPS-induced footpad swelling reaction was suppressed in mice treated systemically with a natural mixture of IFN- $\alpha\beta$  or rIFN- $\alpha$  after local LPS challenge (45). The anti-inflammatory effect of systemic IFN- $\alpha\beta$  on LPS-induced immune responses is suggested to be mediated directly through down-regulation of TNF- $\alpha$  or more indirectly through regulation of one or several molecules that mediate the inhibitory effect. However, our results clearly demonstrate a proinflammatory effect of IFN- $\alpha\beta$ . This apparent discrepancy might relate to the timing of IFN- $\alpha\beta$  relative to LPS administration. In the above cited study, IFN- $\alpha\beta$  was given either immediately after or just before LPS challenge, which may not have allowed sufficient time for IFN- $\alpha\beta$  to exert a priming activity. Also the environment in which the animals are maintained may critically influence the responsiveness to IFN- $\alpha\beta$ .

Based on their immune modulatory effects IFNs are currently being used as treatment against a wide variety of diseases (46, 47). Thus IFN- $\alpha$  is used to treat patients with certain cancers and chronic viral diseases, whereas IFN- $\beta$  is used to treat patients suffering from the autoimmune disease multiple sclerosis. In general, these treatments have beneficial effects but pathology induced by IFN treatment has also been reported (46, 47). In view of our results, it is possible that IFN treatment could influence susceptibility to LPS-induced pathology in patients undergoing IFN treatment, but this obviously needs to be explored.

In conclusion, our results demonstrate that like LPS and bacterial DNA, virus infections may cause rapid sensitization of mice to LPS-induced shock. Consequently, early virus-induced sensitization to LPS may be regarded as a virus-induced analogue of the generalized Shwartzman reaction. However, while LPS sensitizes mice through NK cell production of IFN- $\gamma$ , viruses act primarily through induction of IFN- $\alpha\beta$ . To our knowledge in vivo priming by IFN- $\alpha\beta$  for production of TNF- $\alpha$  has not been reported before.

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## References

- Glauser, M. P., G. Zanetti, J. D. Baumgartner, and J. Cohen. 1991. Septic shock: pathogenesis. *Lancet* 338:732.
- Heinzel, F. P. 1990. The role of IFN- $\gamma$  in the pathology of experimental endotoxemia. *J. Immunol.* 145:2920.
- Doherty, G. M., J. R. Lange, H. N. Langstein, H. R. Alexander, C. M. Buresh, and J. A. Norton. 1992. Evidence for IFN- $\gamma$  as a mediator of the lethality of endotoxin and tumor necrosis factor- $\alpha$ . *J. Immunol.* 149:1666.
- Rothe, J., W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann. 1993. Mice lacking the tumor necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* 364:798.
- Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and inflammatory responses in TNF- $\alpha$ -deficient mice: a critical requirement for TNF- $\alpha$  in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J. Exp. Med.* 184:1397.
- Garcia, I., Y. Miyazaki, K. Araki, M. Araki, R. Lucas, G. E. Grau, G. Milon, Y. Belkaid, C. Montixi, and W. Lesslauer. 1995. Transgenic mice expressing high levels of soluble TNF-R1 fusion protein are protected from lethal septic shock and cerebral malaria, and are highly sensitive to *Listeria monocytogenes* and *Leishmania major* infections. *Eur. J. Immunol.* 25:2401.
- Kohler, J., D. Heumann, G. Garotta, D. LeRoy, S. Bailat, C. Barras, J. D. Baumgartner, and M. P. Glauser. 1993. IFN- $\gamma$  involvement in the severity of Gram-negative infections in mice. *J. Immunol.* 151:916.
- Silva, A. T., and J. Cohen. 1992. Role of interferon- $\gamma$  in experimental Gram-negative sepsis. *J. Infect. Dis.* 166:331.
- Car, B. D., V. M. Eng, B. Schnyder, L. Ozmen, S. Huang, P. Gallay, D. Heumann, M. Aguet, and B. Ryffel. 1994. Interferon- $\gamma$  receptor deficient mice are resistant to endotoxic shock. *J. Exp. Med.* 179:1437.
- Stout, R. D. 1993. Macrophage activation by T cells: cognate and non-cognate signals. *Curr. Opin. Immunol.* 5:398.
- Williams, J. G., G. J. Jurkovich, G. B. Hahnel, and R. V. Maier. 1992. Macrophage priming by interferon  $\gamma$ : a selective process with potentially harmful effects. *J. Leukocyte Biol.* 52:579.
- Tsujimoto, M., Y. K. Yip, and J. Vilcek. 1986. Interferon- $\gamma$  enhances expression of cellular receptors for tumor necrosis factor. *J. Immunol.* 136:2441.
- Liew, F. Y., Y. Li, and S. Millott. 1990. Tumor necrosis factor- $\alpha$  synergizes with IFN- $\gamma$  in mediating killing of *Leishmania major* through the induction of nitric oxide. *J. Immunol.* 145:4306.
- Ozmen, L., M. Pericin, J. Hakimi, R. A. Chizzonite, M. Wysocka, G. Trinchieri, M. Gately, and G. Garotta. 1994. Interleukin-12, interferon- $\gamma$ , and tumor necrosis factor- $\alpha$  are the key cytokines of the generalized Shwartzman reaction. *J. Exp. Med.* 180:907.
- Billiau, A. 1988.  $\gamma$ -Interferon: the match that lights the fire? *Immunol. Today* 9:37.
- Fehniger, T. A., H. Yu, M. A. Cooper, K. Suzuki, M. H. Shah, and M. A. Caligiuri. 2000. Cutting edge: IL-15 costimulates the generalized Shwartzman reaction and innate immune IFN- $\gamma$  production in vivo. *J. Immunol.* 164:1643.
- Cowdery, J. S., J. H. Chace, A. K. Yi, and A. M. Krieg. 1996. Bacterial DNA induces NK cells to produce IFN- $\gamma$  in vivo and increases the toxicity of lipopolysaccharides. *J. Immunol.* 156:4570.
- Heremans, H., C. Dillen, J. van Damme, and A. Billiau. 1994. Essential role for natural killer cells in the lethal lipopolysaccharide-induced Shwartzman-like reaction in mice. *Eur. J. Immunol.* 24:1155.
- Nansen, A., J. P. Christensen, O. Marker, and A. R. Thomsen. 1997. Sensitization to lipopolysaccharide in mice with asymptomatic viral infection: role of T cell-dependent production of interferon- $\gamma$ . *J. Infect. Dis.* 176:151.
- Nguyen, K. B., and C. A. Biron. 1999. Synergism for cytokine-mediated disease during concurrent endotoxin and viral challenges: roles for NK and T cell IFN- $\gamma$  production. *J. Immunol.* 162:5238.
- Bro-Jorgensen, K., and S. Knudtzon. 1977. Changes in hemopoiesis during the course of acute LCM virus infection in mice. *Blood* 49:47.
- Schattnr, A., A. Meshorer, and D. Wallach. 1983. Involvement of interferon in virus-induced lymphopenia. *Cell Immunol.* 79:11.
- Gresser, I., M. G. Tovey, C. Maury, and M. T. Bandu. 1976. Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. II. Studies with herpes simplex, Moloney sarcoma, vesicular stomatitis, Newcastle disease, and influenza viruses. *J. Exp. Med.* 144:1316.
- Muller, U., U. Steinhoff, L. F. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264:1918.
- Thomsen, A. R., and C. J. Phau. 1993. Influence of host genes on the outcome of murine lymphocytic choriomeningitis virus infection. In *The Arenaviridae*. M. S. Salvato, ed. Plenum Press, New York, pp. 199–224.
- Pace, J. L., S. W. Russell, P. A. LeBlanc, and D. M. Murasko. 1985. Comparative effects of various classes of mouse interferons on macrophage activation for tumor cell killing. *J. Immunol.* 134:977.
- Zhou, A., Z. Chen, J. A. Rummage, H. Jiang, M. Kolosov, I. Kolosova, C. A. Stewart, and R. W. Leu. 1995. Exogenous interferon- $\gamma$  induces endogenous synthesis of interferon- $\alpha$  and - $\beta$  by murine macrophages for induction of nitric oxide synthase. *J. Interferon Cytokine Res.* 15:897.
- Korngold, R., K. J. Blank, and D. M. Murasko. 1983. Effect of interferon on thoracic duct lymphocyte output: induction with either poly I:poly C or vaccinia virus. *J. Immunol.* 130:2236.
- Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman. 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. *Proc. Natl. Acad. Sci. USA* 58:1004.
- Marker, O., and M. Volkert. 1973. Studies on cell-mediated immunity to lymphocytic choriomeningitis virus in mice. *J. Exp. Med.* 137:1511.

31. Brunda, M. J., D. Bellantoni, and V. Sulich. 1987. In vivo anti-tumor activity of combinations of interferon- $\alpha$  and interleukin-2 in a murine model. Correlation of efficacy with the induction of cytotoxic cells resembling natural killer cells. *Int. J. Cancer* 40:365.
32. Brunda, M. J., D. Rosenbaum, and L. Stern. 1984. Inhibition of experimentally-induced murine metastases by recombinant  $\alpha$  interferon: correlation between the modulatory effect of interferon treatment on natural killer cell activity and inhibition of metastases. *Int. J. Cancer* 34:421.
33. Welsh, R. M., C. A. Biron, J. F. Bukowski, K. W. McIntyre, and H. Yang. 1984. Role of natural killer cells in virus infections of mice. *Surv. Synth. Pathol. Res.* 3:409.
34. Orange, J. S., and C. A. Biron. 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN- $\gamma$  production and antiviral defense: studies of natural killer and T cell responses in contrasting viral infections. *J. Immunol.* 156:1138.
35. Tough, D. F., and J. Sprent. 1996. Viruses and T cell turnover: evidence for bystander proliferation. *Immunol. Rev.* 150:129.
36. Ernst, D. N., W. O. Weigle, D. J. Noonan, D. N. McQuitty, and M. V. Hobbs. 1993. The age-associated increase in IFN- $\gamma$  synthesis by mouse CD8<sup>+</sup> T cells correlates with shifts in the frequencies of cell subsets defined by membrane CD44, CD45RB, 3G11, and MEL-14 expression. *J. Immunol.* 151:575.
37. Yoshimoto, T., H. Okamura, Y. I. Tagawa, Y. Iwakura, and K. Nakanishi. 1997. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon- $\gamma$  production from activated B cells. *Proc. Natl. Acad. Sci. USA* 94:3948.
38. Diefenbach, A., H. Schindler, N. Donhauser, E. Lorenz, T. Laskay, J. MacMicking, M. Rollinghoff, I. Gresser, and C. Bogdan. 1998. Type 1 interferon (IFN $\alpha/\beta$ ) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* 8:77.
39. Brunda, M. J., D. Tarnowski, and V. Davatelis. 1986. Interaction of recombinant interferons with recombinant interleukin-2: differential effects on natural killer cell activity and interleukin-2-activated killer cells. *Int. J. Cancer* 37:787.
40. Gutierrez-Ramos, J. C., and H. Bluethmann. 1997. Molecules and mechanisms operating in septic shock: lessons from knockout mice. *Immunol. Today* 18:329.
41. Brouckaert, P., and W. Fiers. 1996. Tumor necrosis factor and the systemic inflammatory response syndrome. In *Pathology of Septic Shock*. E. T. Rietschel and H. Wagner, eds. Springer, Berlin, pp. 167-187.
42. Peschke, T., A. Bender, M. Nain, and D. Gerns. 1993. Role of macrophage cytokines in influenza A virus infections. *Immunobiology* 189:340.
43. Tzung, S. P., T. C. Mahl, P. Lance, V. Andersen, and S. A. Cohen. 1992. Interferon- $\alpha$  prevents endotoxin-induced mortality in mice. *Eur. J. Immunol.* 22:3097.
44. Bartholdy, C., A. Nansen, O. Marker, and A. R. Thomsen. 1999. Soluble tumour necrosis factor (TNF)-receptor levels in serum as markers of anti-viral host reactivity. *Clin. Exp. Immunol.* 116:299.
45. Heremans, H., R. Dijkmans, H. Sobis, F. Vandekerckhove, and A. Billiau. 1987. Regulation by interferons of the local inflammatory response to bacterial lipopolysaccharide. *J. Immunol.* 138:4175.
46. Belardelli, F., and I. Gresser. 1996. The neglected role of type I interferon in the T-cell response: implications for its clinical use. *Immunol. Today* 17:369.
47. Weinstock-Guttman, B., R. M. Ransohoff, R. P. Kinkel, and R. A. Rudick. 1995. The interferons: biological effects, mechanisms of action, and use in multiple sclerosis. *Ann. Neurol.* 37:7.